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## PH.D. THESIS

# LIPOPOLYSACCHARIDE-MEDIATED NEUTROPHIL VEGF-A RELEASE IS MODULATED BY CANNABINOID RECEPTOR ACTIVATION


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**SOMMARIO**

**Introduzione**: I neutrofili (PMN) sono cellule dell'immunità innata coinvolte nei processi infiammatori e nelle infezioni. Sia l'angiogenesi infiammatoria che quella tumorale sono modulate da una produzione sequenziale e coordinata di fattori angiogenici quali fattori di crescita endoteliale vascolare (VEGF), angiopoietine (ANGPT), fattore di crescita degli epatociti (HGF) e chemochine. Questi fattori sono prodotti da diverse cellule immunitarie, tra cui i PMN. L'attivazione del recettore dei cannabinoidi di tipo 1 ($CB_1$) e -2 ($CB_2$) è stata suggerita come una nuova strategia per modulare l'angiogenesi *in vitro* e *in vivo*.

**Scopo:** Valutare se l'attivazione di $CB_1$ e $CB_2$ da parte degli agonisti del CB modulasse l'attività angiogenica dei PMN indotta dal lipopolisaccaride (LPS)

**Metodi:** I PMN altamente purificati sono stati isolati da buffy coat di donatori sani. Le cellule sono state stimolate con agonisti/antagonisti $CB_1$ e $CB_2$ da soli e/o in combinazione con LPS. La concentrazione dei fattori angiogenici nei surnatanti cellulari è stata valutata mediante ELISA. La modulazione dei marcatori di attivazione dei PMN da parte degli agonisti CB è stata valutata mediante citofluorimetria. L'angiogenesi *in vitro* è stata valutata come formazione dei tubuli mediante microscopia ottica. La permeabilità endoteliale è stata valutata mediante un test di permeabilità vascolare *in vitro*.

**Risultati:** L'LPS induce la secrezione di VEGF-A, CXCL8 e HGF dai PMN. La preincubazione dei PMN con agonisti $CB_1$ e $CB_2$ inibisce il rilascio di VEGF-A indotto da LPS, ma non influenza la produzione di CXCL8 e HGF. Gli effetti degli agonisti CB sul rilascio di VEGF-A indotti dalla LPS sono annullati dalla preincubazione con i rispettivi antagonisti. Gli agonisti CB modulano l'angiogenesi *in vitro* e la permeabilità endoteliale indotta dai supernatanti di PMN attivati con LPS attraverso la riduzione del VEGF-A.

**Conclusioni:** I neutrofili svolgono un ruolo centrale nel controllo delle infezioni batteriche e nella sepsi. Quest'ultima condizione è associata ad un aumento dei livelli circolanti di VEGF-A. Abbiamo dimostrato gli agonisti CB inibiscono il rilascio di VEGF-A da PMN attivati da LPS. Questi risultati suggeriscono che gli agonisti CB potrebbero rappresentare una nuova strategia terapeutica nei pazienti con sepsi.

**ABSTRACT**

**Background:** Neutrophils (PMNs) are innate immune cells with primary roles in inflammation and in host defense against infections. Both inflammatory and tumor angiogenesis are modulated by a sequential, coordinated production of angiogenic factors such as vascular endothelial growth factors (VEGFs), angiopoietins (ANGPTs), hepatocyte growth factor (HGF), and chemokines. These factors are produced by several immune cells, including PMNs. Activation of cannabinoid receptor type-1 ($CB_1$) and -2 ($CB_2$) has been suggested as a new strategy to modulate *in vitro* and *in vivo* angiogenesis.

**Objective:** We sought to investigate whether activation of $CB_1$ and $CB_2$ by CB agonists modulate lipopolysaccharide (LPS)-mediated angiogenic activity of human PMNs.

**Methods:** Highly purified PMNs were isolated from buffy coats of healthy donors. Cells were stimulated with $CB_1$ and $CB_2$ agonists/antagonists alone and/or in combination with LPS. Angiogenic factors in cell-free supernatants were measured by ELISA. The modulation of activation markers of PMNs by CB agonists was evaluated by flow cytometry. Angiogenesis *in vitro* was measured as tube formation by optical microscopy. Endothelial cell permeability was assessed by an *in vitro* vascular permeability assay.

**Results:** LPS-activated PMNs released VEGF-A, CXCL8 and HGF. Preincubation of PMNs with low concentrations of $CB_1$ and $CB_2$ agonists inhibited VEGF-A release induced by LPS, but did not affect CXCL8 and HGF production. The effects of CB agonists on VEGF-A release induced by LPS were reversed by preincubation with CB antagonists. CB agonists modulated *in vitro* angiogenesis and endothelial permeability induced by supernatants of LPS-activated PMNs through the reduction of VEGF-A.

**Conclusions:** Neutrophils play a central role in the control of bacterial infections and in the outcome of sepsis. The latter condition is associated with an increase in circulating levels of VEGF-A. We demonstrated that low concentrations of CB agonists inhibit VEGF-A release from LPS-activated PMNs. These results suggest that CB agonists might represent a novel therapeutic strategy in patients with sepsis.

## INTRODUCTION

*Neutrophils*

Discovered at the end of the nineteenth century neutrophils (PMNs) are the most abundant white blood cells in mammals. PMNs have a pivotal role in antimicrobial immunity during acute inflammatory response, particularly in the clearance of extracellular pathogens[1]. In the last decades this view has been challenged due to recent evidences indicating that neutrophils survival time is significantly longer than the lifespan usually reported (< 1 day)[2] and they produce a variety of cytokines and chemokines[3], lipid mediators, angiogenic factors[4] and neutrophil extracellular traps (NETs)[5]. There is now evidence that PMNs play a role in several chronic inflammatory disorders[6], cardiovascular diseases[7], asthma[8, 9], tumors[4, 10], sepsis[11, 12] and parasitic diseases[13].

To exert their functions, PMNs express a plethora of immunoreceptors, including Toll like receptors (TLRs)[14] and endocannabinoids receptors[15]. In particular, human PMNs express TLR4 and produce several proinflammatory mediators following the binding to its ligand lipopolysaccharide (LPS)[3, 4], the major component of the outer membrane of Gram-negative bacteria[14]. LPS is recognized as the most potent microbial mediator implicated in the pathogenesis of sepsis and septic shock[16].

*Endocannabinoid system and angiogenesis*

The endocannabinoid system consists of endogenous lipids that exert their functions by activating two 7-transmembrane G-protein-coupled receptors, cannabinoid receptor type-1 ($CB_1$) and cannabinoid receptor type-2 ($CB_2$)[17-19]. $CB_1$ and $CB_2$ are expressed in human PMNs[20, 21] and their activation by endogenous cannabinoids (EC) inhibits neutrophil chemotaxis[21].

Angiogenesis, the formation of new blood vessels, is a complex process requiring a finely tuned balance between stimulatory and inhibitory signals[22, 23]. During adulthood, angiogenesis plays a role in chronic inflammation[24], tissue injury or remodeling[25], cancer[26] and sepsis[27]. Vascular endothelial growth factors (VEGFs)[28], certain chemokines such as CXCL8[29], angiopoietin 1 (ANGPT1) and ANGPT2[30], and hepatocyte growth factor (HGF)[31] are major pro-angiogenic molecules acting on specific receptors expressed on blood endothelial cells.

There is compelling evidence that VEGF is increased in human[32] and experimental sepsis[33, 34]. The imbalance of angiogenic factors and their receptors, including

VEGF/VEGF receptor pathway, has been shown to predict poor prognosis in sepsis[40]. PMNs are an essential arm of the innate immune response against sepsis[11, 12] and, like several other immune cells, are an important source of several pro-angiogenic mediators[4, 10, 41-43]. Several studies have shown that $CB_1$ and $CB_2$ receptor activation can modulate angiogenesis *in vitro* and *in vivo*[44-47].

To the best of our knowledge, there are no published data on the effects of $CB_1/CB_2$ receptor activation on the release of angiogenic factor for human PMNs induced by TLR4 engagement. In this study we demonstrate that activation of $CB_1$ and $CB_2$ inhibits VEGF-A release from LPS stimulated PMNs and that neutrophil VEGF-A was directly angiogenic *in vitro* by stimulating endothelial permeability and tubule formation. Therefore, the activation of CB receptors on PMNs might be a novel strategy to modulate certain aspects of neutrophil-assisted angiogenesis in patients with sepsis.

## MATERIALS AND METHODS

*Reagents*

The following were purchased: L-glutamine, antibiotic-antimycotic solution (10,000 IU/ml penicillin, 10 mg/ml streptomycin, and 25 µg/ml amphotericin B), detoxified LPS (from *E. coli* serotype 0111:B4), N-formylmethionine-leucyl-phenylalanine (fMLF), (Sigma-Aldrich, Milan, ITA). RPMI and fetal calf serum (FCS, endotoxin level <0.1EU/ml) (MP Biomedicals Europe, Illkirch, France). Target-specific primers for *VEGF-A*$_{165}$, *CXCL8* and *GAPDH* were produced and purified by Custom Primers (Life Technologies, Milan, Italy). All other reagents were from Carlo Erba (Milan, Italy).

*Isolation and purification of PMNs*

The study protocol involving the use of human blood cells was approved by the Ethics Committee of the University of Naples Federico II (Protocol number 301/12), and written informed consent was obtained from blood donors according to the principles expressed in the Declaration of Helsinki. Granulocytes were isolated from buffy coats of healthy donors ($HBsAg^-$, $HCV^-$, and $HIV^-$) obtained from a leukapheresis unit. Leukocytes were separated from erythrocytes by dextran sedimentation. PMNs were purified by Histopaque-1077 (Sigma-Aldrich,) density gradient centrifugation (400 × g for 20 min at 22˚C), followed by Percoll (Sigma-Aldrich) (65%) density gradient centrifugation (660 × g for 20 min at 22˚C), as previously described[10]. Finally, PMNs were purified (to reach >99% purity) by positive elimination of all contaminating cells using the EasySep Neutrophil Enrichment Kit (StemCell Technologies, Vancouver, Canada)[48]. These cells were >99% neutrophils as evaluated by flow-cytometric analysis with the following antibodies: anti-CD3, anti-CD14, anti-CD15, anti-CD11b, anti-CCR3 (Miltenyi Biotec, Germany), anti-CD62L (L-selectin) (BD Biosciences, USA), and anti-CD66b (Biolegend, San Diego, CA, USA). Samples were analyzed on the MACSQuant Analyzer 10 (Miltenyi Biotec) and in the FlowJo software, v.10. Doublets and debris were excluded from the analysis. Data were expressed as a percentage of positive cells or median fluorescence intensity[49] (Fig. 1). PMNs were suspended ($5x10^6$ cells/ml) in complete medium (RPMI 1640 containing 5% FCS, 2 mM L-glutamine and 1% antibiotic-antimycotic solution) and incubated in different plates (Falcon, Becton Dickinson, Franklin Lakes, NJ) at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air. After 30 min of rest, the cells were used for the experiments.

*Cell cultures*

PMNs, suspended in complete medium, were preincubated (37°C, 20 min) with or without increasing concentrations ($10^{-4}$-$10^{3}$nM) of ACEA ($CB_1$ agonist) and JWH-133 ($CB_2$ agonist) (Tocris Bioscience, UK)[50, 51] and then stimulated (from 30 min to 16 hrs at 37°C) with LPS (100 ng/ml) or fMLF (50 nM). In selected experiments, PMNs were preincubated (37°C, 10 min) with AM-251 ($CB_1$ antagonist) or AM-630 ($CB_2$ antagonist) (Tocris Bioscience) (500 nM)[50, 51] before the addition of CB agonists and LPS. At the end of the experiment, the supernatants were harvested, centrifuged (300 g, 5 min, 4°C) and stored at –80°C for subsequent experiments.

*ELISA assays*

Cytokine concentrations in supernatant and in cellular lysates were measured using commercially available ELISA kits for VEGF-A, CXCL8 and HGF (R&D System, Minneapolis, MN, USA). The results were normalized for the number of cells per well. Cytokine release was expressed as $R–R_b$ pg/$10^6$ cells where R is the release in samples treated with LPS or fMLF alone or in combination with CB agonists or antagonists, $R_b$ is the release in unstimulated or CB agonists and antagonists samples. The percentage of the maximal response calculated as $(R–R_b)/(R_{max}–R_b)$ x 100, where R is the release in samples treated with the combination of LPS plus CB agonists or antagonists, $R_b$ is the release in unstimulated samples and $R_{max}$ is the release in samples stimulated with LPS alone.

*mRNA extraction and quantitative PCR (qPCR) analysis*

Total RNA was isolated with RNeasy plus Minikit (Qiagen, Italy) following manufacturer's instructions. RNA quality and integrity was estimated with 2100 Agilent Bionalyzer. Total mRNA was reverse-transcribed (high capacity CDNA RT, Life Technologies) and qPCR was carried out in Master Cycler realplex (Eppendorf, Milan, Italy) using iTaq$^{tm}$ Universal SYBR® Green Supermix (Bio-Rad, Hercules,CA, USA). *GAPDH* was used as housekeeping gene to normalize Ct (cycle threshold) values using the $2^{-\Delta Ct}$ formula. The following primer pairs were used: *GAPDH* forward, 5'-CACCATCTTCCAGGAGCG-3', reverse, 5'-GACTCCACGACGTACTCAGC-3'; *CXCL8* forward, 5'-CTGGCCCTGGCTCTCTTG-3', reverse, 5'-CCTTGGCAAAACTGCACCTT-3'; *VEGF-$A_{165}$* forward, 5'-GCCTTGCCTTGCTGCTCTAC-3', reverse, 5'- TGATTCTGCCCTCCTCCTTCTG-3'.

*Apoptosis assay*

Purified neutrophils ($5x10^5$ cells/well) were cultured in complete medium alone or in presence of LPS (100 ng/ml) or fMLF (50 nM) for 16 hrs at 37°C. Neutrophils were stained with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) according to the protocol provided by the manufacturer (Miltenyi Biotech). Quantification was performed on a MACS Quant flow cytometer (Miltenyi Biotech). Live cells were assumed to be double-negative annexin $V^-PI^-$cells. Analysis was performed by means of FlowJo v.10.

*Flow cytometry*

For activation experiments, PMNs were kept in complete medium for 30 min stimulated with CB agonists (100 nM) and LPS (100 ng/ml) or their combination for 30 min. Cells were washed and incubated in staining buffer (phosphate buffered saline [PBS], 10% human AB serum, 0.05% $NaN_3$) at 4°C for 20 min with the following antibodies: allophycocyanin (APC)-conjugated anti-human CD66b (Clone REA306from Miltenyi Biotech) and FITC-conjugated anti-human CD62L (Clone REAL163 from Miltenyi Biotech). Then, cells were washed in washing buffer (PBS, 0.2% BSA, 0.05% NaN3) and fixed in 2% paraformaldehyde before acquisition on MACSQuant Analyzer 10 (Miltenyi Biotech) and analyzed using FlowJo v10. Doublets, debris (identified based on forward and side scatter properties), and dead cells (identified with Zombie Violet Fixable Viability Kit; Biolegend) were excluded from the analysis. Data are expressed as a percentage of positive cells or median fluorescence intensity[49].

*In vitro Matrigel angiogenesis assay*

Bovine aortic endothelial cells (BAEC) were trypsinized and resuspended in RPMI 1640 containing 10% FCS, 2 mM L-glutamine and 1% antibiotic-antimycotic solution. 24-well plates coated (1 hr, 37°C) with 80 µl of Matrigel (Sigma-Aldrich) according to the manufacturer's instructions. BAEC ($6 \times 10^4$) were seeded on Matrigel Matrix in 350 µl of medium and after 1 hr of adherence at 37°C we added 150 µl (30% of total volume) of supernatants of PMNs activated by medium alone, CB agonists, CB antagonists, LPS, LPS with CB agonists, LPS with CB antagonists plus agonists or VEGF-$A_{165}$ (20 ng/ml) as positive control, for 6 hrs at 37°C. In selected experiments supernatants of unstimulated or LPS-activated PMNs were treated 1 hr at 37°C with a neutralizing antibody anti-VEGF-$A_{165}$ (1 µg/ml). Tube formation was evaluated by optical microscopy using a Nikon Eclipse TS100 with Nikon Digital Sight DS-L1 (Nikon Ltd., London, England, x2.8 magnification). Digital

image analysis was performed by Fiji Image software. For quantification, two parameters were analyzed: tubule length and number of tubules[52].

*In vitro vascular permeability assay*

Endothelial cell permeability was assessed by *in vitro* vascular permeability assay kit (Life Technologies). BAEC were seeded onto collagen-coated Transwell filters (1μm pore size) at the density of $3\times10^5$ cells per well with 200 μl of RPMI 1640 (10% FCS) in a 24-well receiver plate with 500 μl of RPMI 1640 (10% FCS), and incubated at 37°C and 5% $CO_2$ for 72 hrs. Starvation was then performed by replacing culture medium with RPMI 0.5% FCS for 18 hrs at 37°C, 5% $CO_2$. BAEC were then stimulated (6 hrs, 37°C) with 30 % of supernatants of PMNs activated by medium alone (containing 10% FCS), CB agonists, CB antagonists, LPS, LPS with CB agonists and or LPS with CB antagonists plus agonists. In selected experiments supernatants of unstimulated and LPS-activated PMNs were treated 1 hr at 37°C with a neutralizing antibody anti-VEGF-$A_{165}$ (1 μg/ml). To evaluate vascular permeability, a high molecular weight FITC-Dextran was added on top of the cells, allowing the fluorescent molecules to pass through the endothelial cell monolayer at a rate proportional to the monolayer's permeability. The extent of permeability was determined by measuring the FITC fluorescence (485 nm excitation and 535 nm emission) using EnSpire Multimode Plate Reader (Perkin Elmer, Boston,MA, USA) and Relative Fluorescent Unit (RFU) was calculated.

*Statistical analysis*

The data are expressed as mean values ± SD of the indicated number of experiments. Statistical analysis was performed in Prism 6 (GraphPad Software). Statistical analysis was performed by Student's T-test or one-way analysis of variance followed by Dunnett's test (when comparison was made against a control) or Bonferroni's test (when comparison was made between each pair of groups) by means of Analyse-it for Microsoft Excel, version 2.16 (Analyse-it Software, Ltd.). Statistically significant differences were accepted when the *p* value was at least $\leq 0.05$.

## RESULTS

*Effects on $CB_1$ and $CB_2$ agonists on the release of angiogenic factors from LPS-activated PMNs*

Upon cellular activation, PMNs express and release several angiogenic factors such as VEGF-A, CXCL8[4, 53] and HGF[54-57]. The results reported in Table 1 confirm that LPS and fMLF, two PMN activators, induced (after 16 hrs of incubation) the release of VEGF-A, CXCL8 and HGF[4, 21, 54, 58, 59]. The percentage of viable PMNs after LPS or fMLF treatment did not differ from that of untreated cells (Fig. 2).

First, we investigated whether CB agonists could affect the release of VEGF-A from LPS-activated PMNs. PMNs were preincubated with ACEA ($CB_1$ agonist) and JWH-133 ($CB_2$ agonist) and stimulated with LPS. Low concentrations of CB agonists selectively inhibited LPS-induced production of VEGF-A (Fig. 3A-B). JWH-133 was more potent than ACEA in inhibiting VEGF-A production induced by LPS [Log($IC_{50}$) of ACEA: -1.95 ± 0.23 nM *vs.* -2.64 ± 0.26 that of JWH-133] (Fig. 4). Moreover, the inhibitory effects of ACEA and JWH-133 on VEGF-A release by LPS-activated PMNs were reversed by preincubation with the $CB_1$ (AM-251) and $CB_2$ (AM-630) antagonists, respectively (Fig. 3C-D). The reversal reached a maximum of 75% also in experiments in which we incubated the cells with a combination of $CB_1$ and $CB_2$ antagonists before the stimulation with $CB_1$ or $CB_2$ agonists (data not shown). CB agonists and antagonists did not modulate the spontaneous release of VEGF-A (Fig. 3C-D). Interestingly, ACEA and JWH-133 did not modulate LPS-induced production of CXCL8 (Fig. 3E-F) or HGF (Fig. 3G-H).

In order to verify whether the effects of CB agonists were specific for PMNs activated by LPS, we assessed the effects of optimal concentration (100 nM) of ACEA and JWH-133 on the secretion of VEGF-A, CXCL8 and HGF from PMNs stimulated by fMLF, a bacterial product which activates a specific receptor expressed by human PNMs[60, 61]. Figure 5 shows that fMLF induced the release of VEGF-A, CXCL8, ANGPT1 and HGF from PMNs. ACEA and JWH-133 did not modify the production of VEGF-A (Fig. 5A), CXCL8 (Fig. 5B) and HGF (Fig. 5C).

We next evaluated the kinetics of the effects of an optimal concentration (100 nM) of CB agonists on LPS-induced VEGF-A. To this end PMNs were kept in culture with LPS from 30 min up to 16 hrs. Figure 6 shows that the production of VEGF-A (panel A, black columns) and CXCL8 (panel B, black columns) is detected at 30 min and progressively increased up to 16 hrs. The inhibitory effects of ACEA and JWH-133 on VEGF-A release were significant

after 6 hrs and reached a maximum after 16 hrs of incubation with LPS (Fig. 6A, grey and white columns). The intracellular concentrations of VEGF-A were not modified by treatment with CB agonists (data not shown) at all time-points tested. As previously shown (Fig. 6E-F), CB agonists had no effect on CXCL8 release at any time-point tested (Fig. 6B).

The kinetics of VEGF-A inhibition illustrated in Figure 6 suggested that ACEA and JWH-133 could modulate gene expression of VEGF-A. Thus, we examined whether CB agonists affected gene expression of *VEGF-A$_{165}$*, the major isoform expressed in normal tissues and in tumors[62]. Real-time quantitative PCR showed that LPS enhanced mRNA expression of *VEGF-A$_{165}$* and *CXCL8* (Fig. 6C-D, black columns) after 3 hrs of incubation. In addition, ACEA (Fig. 6C, dashed columns) and JWH-133 (Fig. 6C, dotted columns) markedly reduced *VEGF-A$_{165}$* mRNA expression, but had no influence on *CXCL8* gene expression (Fig. 6D).

*Effects of CB$_1$ and CB$_2$ agonists on the expression of CD66b and CD62L in LPS-activated PMNs*

Resting PMNs marginally express the specific granule marker CD66b and highly express the surface receptor for adhesion L-selectin (CD62L)[42]. To determine whether ACEA and JWH-133 influenced LPS-activated human PMNs, we evaluated CD66b and CD62L expression in PMNs by flow cytometry[63, 64]. PMNs were stimulated for 30 min with LPS, with or without CB agonists. PMNs were then stained with antibodies against CD66b and CD62L and evaluated by flow cytometry. LPS induced a rapid increase of CD66b expression (Fig. 7A), and a decrease of CD62L expression (Fig. 7B, and Fig. 8), and an increase in the percentage of CD62L negative cells (Fig. 7C). All these effects were not modified by preincubation with CB$_1$ and CB$_2$ agonists (Fig. 7A-C and Fig. 8).

*CB$_1$ and CB$_2$ agonists impair in vitro angiogenesis*

The results of the experiments previously reported demonstrate that ACEA and JWH-133 inhibited the *in vitro* release of VEGF-A from LPS-activated PMNs. The production of tubular structures is a critical process in angiogenesis[65]. Therefore, we investigated whether supernatants from LPS-activated PMNs are able to induce an angiogenic response *in vitro*. By using the Matrigel assay, we stimulated (6 hrs, 37°C) BAEC with supernatants from PMNs treated with LPS for 16 hrs at 37°C. BAEC stimulated with vehicle alone or with VEGF-A$_{165}$ were used as negative and positive controls, respectively. The supernatant from LPS-activated PMNs induced tube formation, measured as tubule length and number of tubules, compared to supernatants from PMNs treated with medium alone (Fig. 9A-B, K-L). The optimal

macroscopic angiogenic response was observed in BAEC treated with VEGF-$A_{165}$ (20 ng/ml) (Fig. 10). To assess whether CB agonists affected the tube formation induced by LPS-activated PMNs supernatants, we stimulated BAEC with supernatants of PMNs preincubated with ACEA or JWH-133 and stimulated with or without LPS. PMNs supernatants incubated with CB agonists alone had no effect on tube formation (Fig. 9C-D, K-L). Combination treatment with LPS significantly inhibited angiogenesis. In particular, the presence of ACEA and JWH-133 reduced the angiogenic response of LPS-activated PMNs supernatants measured as tubule length (Fig. 9E-F, K) and number of tubules (Fig. 9E-F, L). These effects were significantly reversed by combined presence in supernatants of $CB_1$ (AM-251) and $CB_2$ (AM-630) antagonists (Fig. 9G-H). Supernatants of PMNs treated only with antagonists did not induce tube formation (Fig. 10). Moreover, the addition of a neutralizing antibody anti-VEGF-$A_{165}$ to supernatants from LPS-activated PMNs reduced the effect of LPS on tube formation (Fig. 9J-L). Anti-VEGF-$A_{165}$ alone had no effect on *in vitro* angiogenesis (Fig. 9I).

*Effects of $CB_1$ and $CB_2$ agonists on endothelial permeability*

To gain mechanistic insight into the role of ACEA and JWH-133 on endothelial permeability, we performed vascular permeability assay by monitoring the leakage of dextran-FITC through a tight monolayer of BAEC[66]. Supernatants of LPS-treated PMNs induced endothelial permeability in BAEC (Fig. 11). Interestingly, ACEA and JWH-133 reduced the vascular permeability induced by supernatants of LPS-treated PMNs. These inhibitory effects were significantly reversed by the presence in supernatants of CB antagonists (Fig. 11). Supernatants of PMNs treated only with agonists and antagonists did not impair endothelial permeability (data not shown). Endothelial permeability induced by supernatants from LPS-activated PMNs was inhibited by treatment with an anti-VEGF-$A_{165}$ antiboby (Fig. 11).

## DISCUSSION

In this study, we demonstrate that low concentrations of $CB_1$ and $CB_2$ agonists (ACEA and JWH-133, respectively) selectively inhibited LPS-induced production of VEGF-A from human PMNs, whereas they did not affect CXCL8 and HGF release. Interestingly, ACEA and JWH-133 inhibited the *in vitro* angiogenic response (i.e., number of tubules and tubule length) induced by LPS-activated PMNs. Finally, supernatants from PMNs treated with CB agonists inhibited the increase of endothelial permeability induced by LPS. These inhibitory effects were presumably due to the reduction of VEGF-A release in PMN supernatants (Fig. 12).

PMNs are crucial players in host defense against invading pathogens and in the outcome of sepsis[11, 12]. There is compelling evidence that VEGF-A, the most important angiogenic factor[62], is increased in human[32] and experimental sepsis[33, 34]. Moreover endocannabinoids are elevated in the sera of patients and animals in septic shock[67] and activation of cannabinoid receptors[68-70] occurs during sepsis. The latter observations suggest that manipulation of the endocannabinoid system may represent an important therapeutic target in managing sepsis and septic shock[68-70].

Endocannabinoids and CB agonists exert anti-inflammatory effects in several *in vitro* and *in vivo* models[20, 45, 71]. In particular, we have previously demonstrated that CB agonists selectively reduced the release of angiogenic factors, (i.e., VEGFs and ANGPTs) from LPS-activated macrophages[43]. In this study we found that low concentrations of ACEA and JWH-133 markedly inhibited the production of VEGF-A induced by LPS-activated PMNs. CB receptors are G protein-coupled receptors and can be desensitized by incubation with high concentrations of agonists[72]. Their desensitization leads the loss of the signal transduction capacity and depends on the exposure time and concentration of the agonist used[72-74]. It is tempting to speculate that high concentrations of ACEA and JWH-133 induced a desensitization of CB receptors causing a reduced inhibition of VEGF-A compared to low doses. Alternatively, high concentrations of agonists can lead to loss of receptor specificity and/or to non-specific activation of other receptors.

Interestingly, unlike the VEGF-A, $CB_1$/$CB_2$ agonists did not influence the release of other angiogenic factors such as CXCL8 and HGF. It is presently unclear the mechanism responsible for this selective inhibitory effect of CB agonists. A possible explanation could be related to the different intracellular localization of the different angiogenic mediators in PMNs. PMNs store a variety of preformed mediators in their secretory granules or in other

compartments[3]. Human PMNs harbor ready-made angiogenic factors, but can also synthesize these mediators *de novo*. These two processes might act synergistically in the context of dysregulated angiogenesis in different pathological conditions[35, 37, 75, 76]. VEGF-A is stored exclusively in the specific (β) granules of human PMNs[77], whereas CXCL8 is localized in a light membrane fraction in LPS-treated neutrophils[78]. Finally, HGF is stored in secretory vesicles and in gelatinase/specific granules[59].

*VEGF-A* and *CXCL8* mRNAs are constitutively expressed by human PMNs[4]. In this study we found that LPS induced, in a time-dependent manner, the release of VEGF-A and CXCL8 from PMNs and increased their mRNA expression. CB agonists inhibited both VEGF-A release and mRNA expression in LPS-stimulated PMNs. By contrast, CB agonists had no effect on the release of CXCL8 from LPS-activated PMNs and the expression of its mRNA. In this study we did not explore the mechanisms responsible for the selective inhibitory effects of CB agonists on the different angiogenic factors (i.e., VEGF-A, CXCL8 and HGF). We would like to speculate that ACEA and JWH-133 might interfere with specific signaling transduction activated by LPS that leads to the production of different mediators. Additional experiments are required to clarify this intriguing finding.

Cannabinoids modulate several aspects of PMN biology[79]. However, the role of the cannabinoid system on PMN migration is controversial. Some studies showed that CB activation did not affect PMN migration, whereas other authors demonstrated that CB agonists induced migration of PMNs toward inflammatory stimuli[21, 79, 80]. Two membrane-bound glycoproteins (i.e., CD66b and CD62L) regulating their adhesive property are involved in neutrophil migration and activation[42]. We found that ACEA and JWH-133 did not alter the expression of CD66b and CD62L in LPS-treated PMNs. The results imply that the inhibitory effect of CB agonists on VEGF-A release was independent from these clusters of differentation.

CB activation was generally associated with anti-inflammatory effects including reduced macrophage and PMN numbers and decreased pro-inflammatory cytokines at the site of infection[43, 67]. In experimental models of moderate sepsis, it has been reported that CB agonists reduced the continuous recruitment of PMNs at the site of infection, while increasing phagocytosis and clearance of bacteria[70]. Conversely, in severe sepsis, where PMN recruitment and function can be injurious, CB activation reduced mortality[81].

The integrity of vascular endothelium and PMNs are key players in microbial infections. LPS, the major component of the outer membrane of Gram-negative bacteria, activate TLR4 on human PMNs to release several proinflammatory and vasoactive

mediators[3, 4]. Guabiraba et al. showed in mice that CB antagonists led to inhibition of angiogenesis due to a reduction of inflammatory cell recruitment and inhibition of inflammatory mediator release such as VEGF[46]. In this study, we demonstrate that CB agonists inhibited the production of the most potent angiogenic (i.e., VEGF-A) and vasoactive mediator[82] from LPS-treated neutrophils. Moreover, the treatment of endothelial cells with supernatants from LPS-stimulated PMNs induced tube formation on growth factor-reduced Matrigel, a critical process in angiogenesis[65, 75] and increased endothelial permeability. These conditions were reverted by pretreatment of PMN supernatants with CB agonists. It is conceivable that CB agonists limit VEGF-A production from LPS-treated PMNs, thus inhibiting angiogenesis and endothelial permeability. This hypothesis is supported by the observation that the addition of an anti-VEGF-A antibody to supernatants from LPS-treated PMNs reduced the ability of LPS to induce both tubule formation and vascular permeability.

Our study has some limitations which have to be pointed out. First, we did not investigate the mechanism responsible for the selective inhibitory effect of CB agonists on VEGF-A release from LPS-activated PMNs. Second, [60, 61]our results show that CB agonists have no effect on fMLF-activated PMNs, differently from LPS-activated cells. Although, fMLP[60, 61] and LPS[3, 4] activate different receptors, we have not investigated the mechanisms underlying the differential effects of CB agonists on PMN activation by these two ligands. Finally, the experiments on endothelial permeability were conducted with BEAC and human PMNs. We cannot exclude that the species differences may have influenced the results.

LPS is recognized as the most potent bacterial product implicated in the pathogenesis of sepsis and septic shock[16]. LPS is a powerful activator of neutrophils that are an essential arm of the innate immune response[3, 4]. Their biology is altered at multiple stages during sepsis [12, 60]. LPS stimulated PMNs release VEGF-A and this factor is directly angiogenic *in vitro* by stimulating endothelial permeability and tubule formation. These findings have important implications in pathophysiological angiogenic processes, including sepsis, where PMNs play a dominant role[12, 83]. In this study, we demonstrate that CB agonists modulate several aspects of neutrophil-assisted angiogenesis mediated by the production of VEGF-A, the most powerful angiogenic factor[62]. VEGF-A levels are increased in human[32] and experimental sepsis[33, 34] and are recognized as a prognostic biomarker for identification of patients with the highest mortality rates[32, 40]. Therefore, our results can have an important impact in the diagnosis and/or treatment of sepsis where neutrophils play a central role.

Further studies are required to determine of therapeutic potential of CB-neutrophil axis in human sepsis[12, 83].

Increasing evidences suggest that LPS and/or neutrophil activation could play a role also in cancer[84-86], in asthma[8, 9][8] and in cardiovascular disorders[7]. All these conditions are associated with altered production of angiogenic factors[33-35, 37]. Therefore, the possibility cannot be excluded that the modulation by cannabinoids of angiogenic factors released by neutrophils might have novel therapeutic implications.

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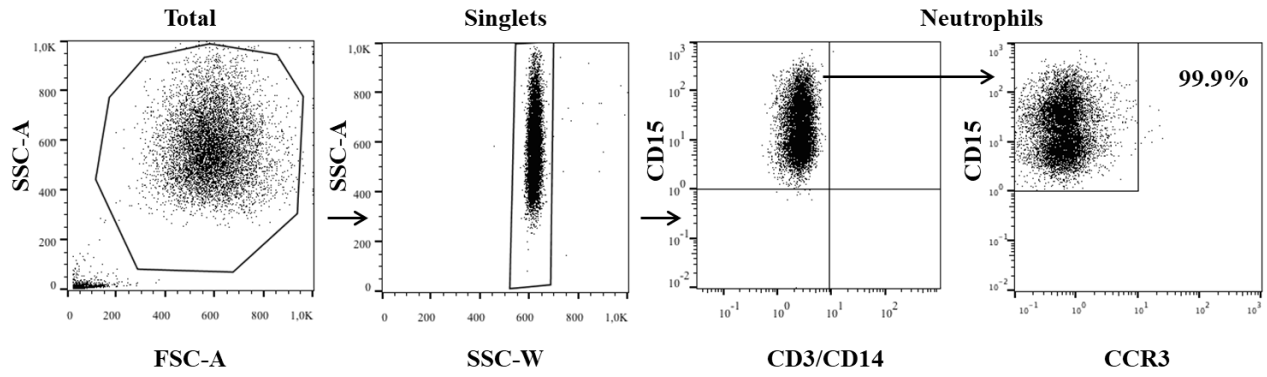


**Figure 1. Phenotypical analysis of purified human PMNs.** PMNs were purified from peripheral blood of healthy donors and stained with the monoclonal antibodies indicated. Flow cytometry plots were gated on live single cells and show forward (FSC) and side scatter (SSC) of EasySep-purified untouched neutrophils, as well as expression of the surface markers CD3, CD14, CCR3 and CD15. This Figure illustrates representative flow cytometric panels of one out of 4 independent experiments.

| | Untreated 1 | LPS | Untreated 2 | fMLF |
|---|---|---|---|---|
| VEGF-A ($pg/10^6$ cells) | 27.0 ± 1.8 | 132.5 ± 6.7* | 35.2 ± 2.9 | 173.7 ± 16.1** |
| CXCL8 ($pg/10^6$ cells) | 30.1 ± 6.5 | 389 ± 38.5* | 28.4 ± 8.4 | 202.1 ± 25.4** |
| HGF ($pg/10^6$ cells) | 130.2 ± 17.6 | 254.3 ± 24.9* | 122.7 ± 12.7 | 315.2 ± 10.5** |

**Table 1 - Effects of LPS and fMLF on the release of VEGF-A, CXCL8 and HGF from human PMNs.** PMNs were stimulated with medium alone (Untreated 1 and Untreated 2), LPS (100 ng/ml) or fMLF (50 nM) for 16 hrs at 37°C. VEGF-A, CXCL8 and HGF concentrations in supernantants were determined by ELISA. Data are the mean ± SD of 8 independent experiments.

*$p < 0.0001$ *vs.* corresponding value of Untreated 1 PMNs.

**$p < 0.0001$ *vs.* corresponding value of Untreated 2 PMNs.

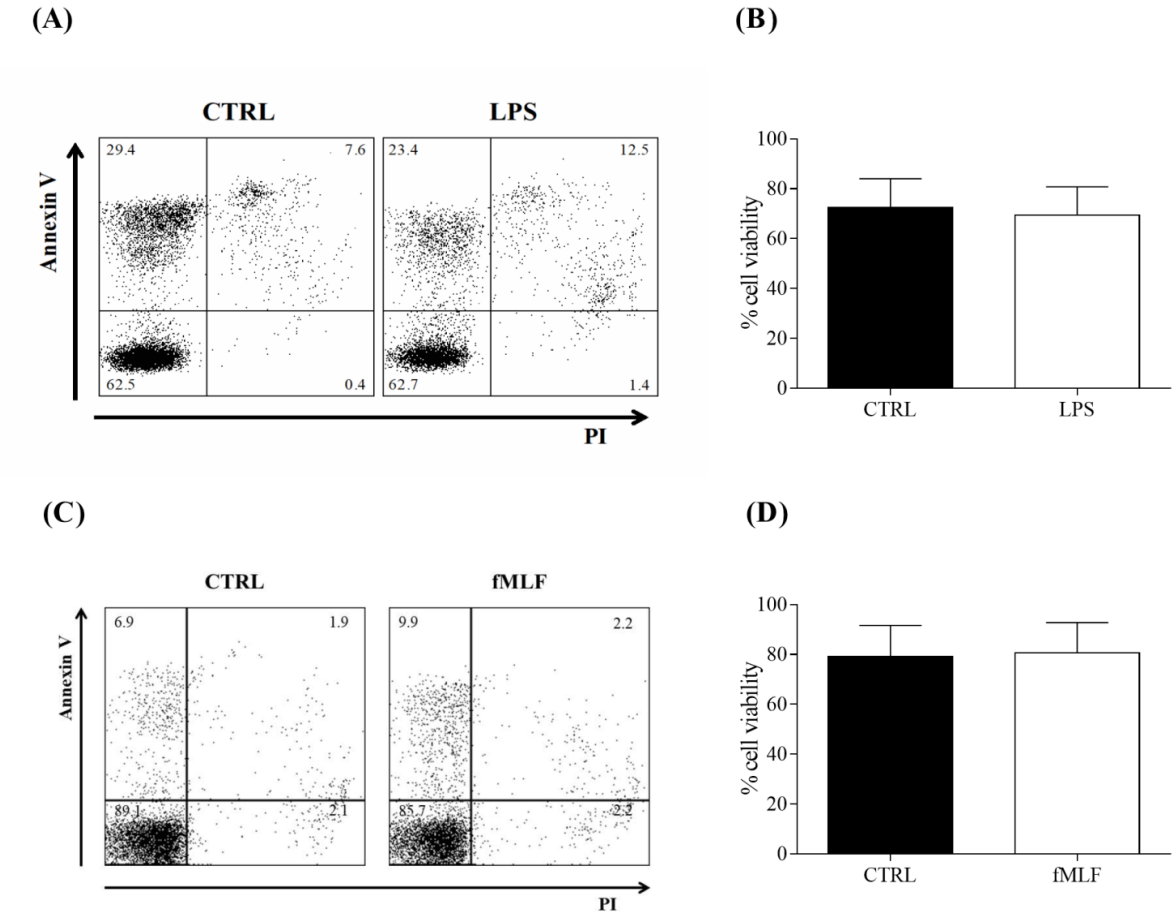


**Figure 2. Apoptosis assay.** PMNs were purified from peripheral blood of healthy donors and incubated with RPMI alone (CTRL), LPS (100 ng/ml) **(A, B)** or fMLF (50 nM) **(C, D)** for 16 hrs at 37°C. Live cells were stained with FITC-conjugated annexin V and PI and analyzed by flow cytometry. Panels **(A, C)** illustrate representative flow cytometric panels of one out of 4 independent experiments. Results are expressed as percentage of live cells **(B, D)**.

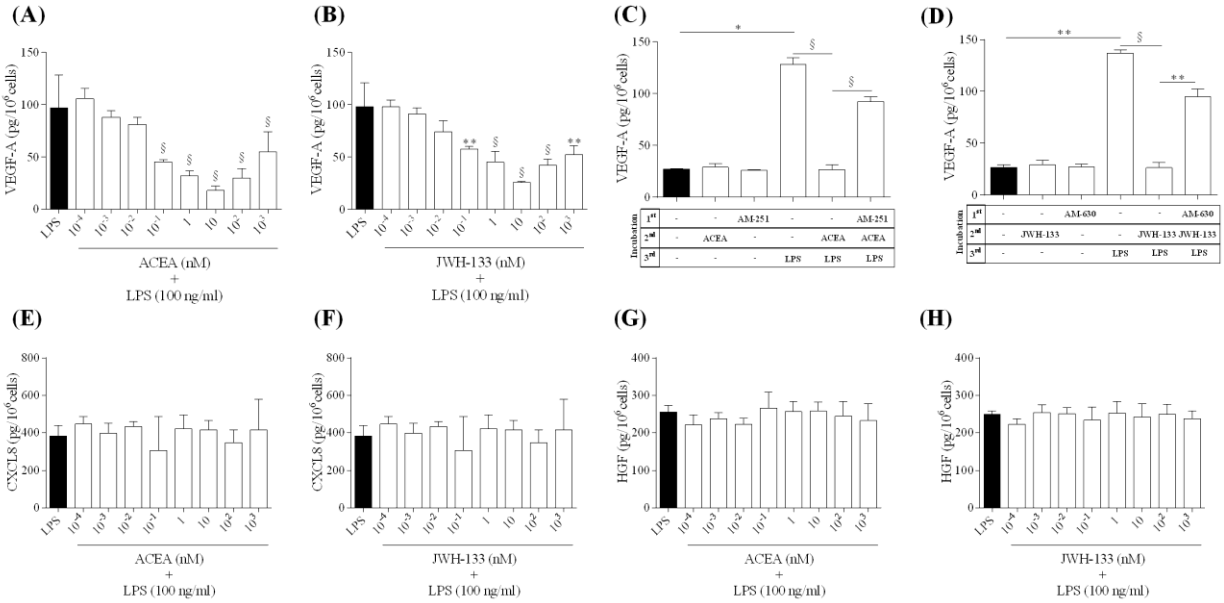


**Figure 3. Effects on $CB_1$ (ACEA) and $CB_2$ (JWH-133) agonists on LPS-induced release of VEGF-A, CXCL8 and HGF from human PMNs.**

**(A, B, E-H)** PMNs were preincubated (20 min, 37°C) with or without indicated concentrations of ACEA or JWH-133 and then stimulated (16 hrs, 37°C) with LPS (100 ng/ml). **(C-D)** PMNs were preincubated (10 min, 37°C) with or without AM-251 (500 nM) **(C)** or AM-630 (500 nM) **(D)**, then stimulated (20 min, 37°C) with ACEA (100 nM) **(C)** or JWH-133 (100 nM) **(D)** and finally stimulated (16 hrs, 37°C) with LPS (100 ng/ml).VEGF-A **(A-D)**, CXCL8 **(E, F)** and HGF **(G, H)** concentrations were determined by ELISA. Data are the mean ± SD of 8 independent experiments with cells from different donors. Cytokine release was expressed as $R–R_b$ pg/$10^6$ cells where R is the release in samples treated with LPS alone or in combination with CB agonists or antagonists, $R_b$ is the release in unstimulated or CB agonists and antagonists samples.

$*p < 0.05$ *vs.*, $**p < 0.001$ and $§p < 0.0001$ *vs.* LPS alone **(A-B)** or respective controls **(C-D)**.

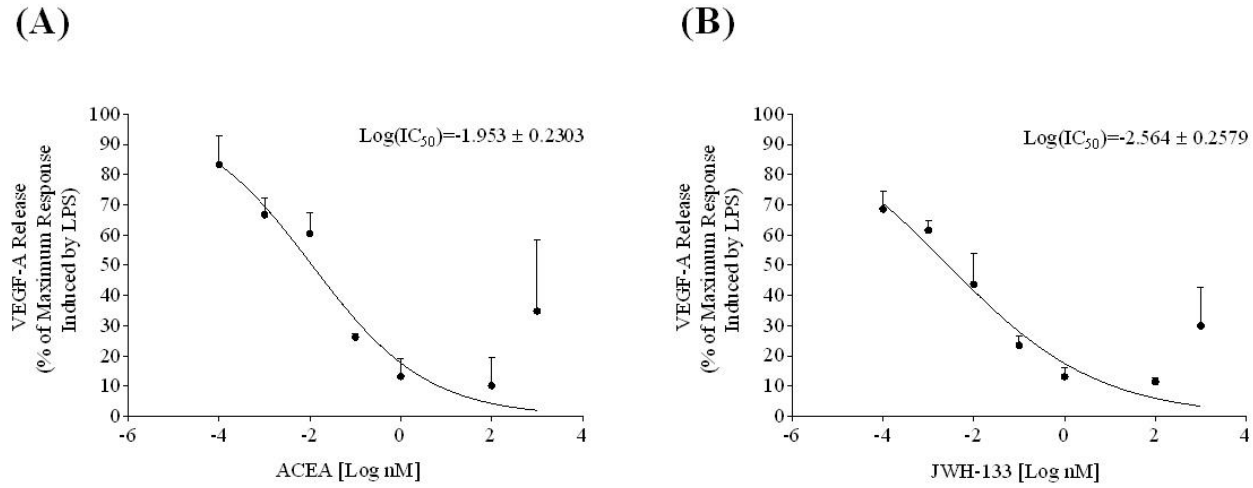


**Figure 4. CB agonists reduce LPS-induced release of VEGF-A from human PMNs.** PMNs were purified from peripheral blood of healthy donors and incubated with or without the indicated concentrations of ACEA **(A)** or JWH-133 **(B)** and then stimulated with LPS (100 ng/ml) for 18 hrs at 37°C. Data are expressed as percent of the maximum response induced by LPS alone for VEGF-A. Data are the mean ± SD of 8 independent experiments with cells from different donors.

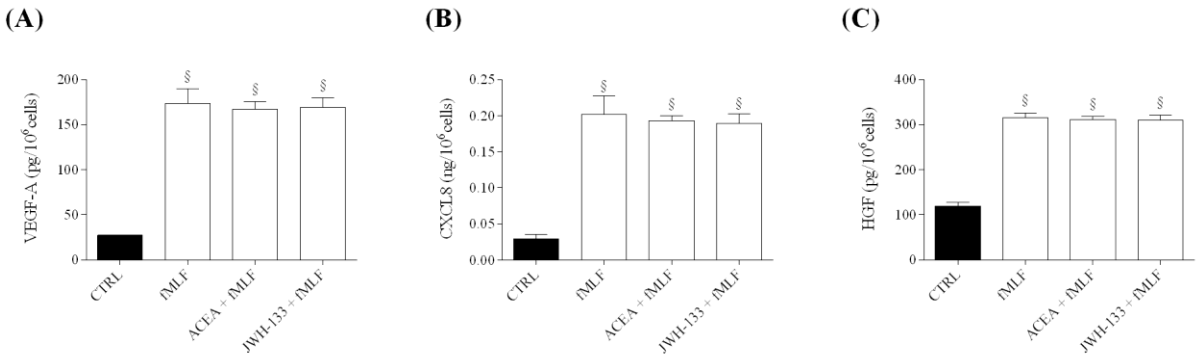


**Figure 5. Effects on $CB_1$ (ACEA) and $CB_2$ (JWH-133) agonists on fMLF-induced release of VEGF-A, CXCL8, and HGF from human PMNs.**

PMNs were preincubated (20 min, 37°C) with or without ACEA (100 nM) or JWH-133 (100 nM) and then stimulated (16 hrs, 37°C) with fMLF (50 nM). VEGF-A **(A),** CXCL8 **(B)** and HGF **(C)** concentrations were determined by ELISA. Data are the mean ± SD of 8 independent experiments with cells from different donors.

*§p* < 0.0001 *vs.* medium alone.

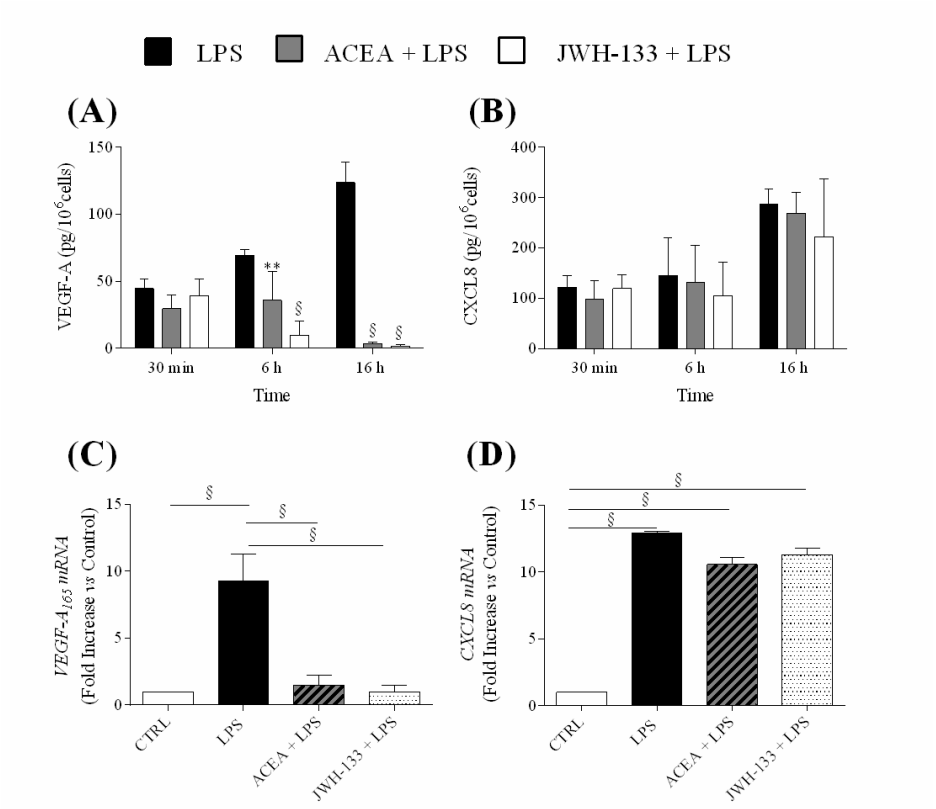


**Figure 6. Kinetics of $CB_1$ (ACEA) and $CB_2$ (JWH-133) agonists on LPS-induced release of VEGF-A and CXCL8 from human PMNs.**

PMNs were stimulated with LPS alone (black column) or in combination with ACEA (100 nM) (grey column) or JWH-133 (100 nM) (white column). VEGF-A **(A)** and CXCL8 **(B)** release was determined by ELISA. **(C-D)** mRNA levels for *VEGF-A*$_{165}$ and *CXCL8* were measured by real-time PCR (see Methods). PMNs were preincubated (20 min, 37°C) with or without ACEA (100 nM) or JWH-133 (100 nM) and then stimulated 3 hrs with LPS (100 ng/ml). Cytokine release was expressed as $R–R_b$ pg/$10^6$ cells where R is the release in samples treated with LPS alone or in combination with CB agonists or antagonists, $R_b$ is the release in unstimulated or CB agonists and antagonists samples. Expression of *VEGF-A*$_{165}$ and *CXCL8* (normalized for *GAPDH*) was expressed as fold changes *vs.* untreated cells. Data are the mean ± SD of 6 experiments.

$^{*}p < 0.05$ *vs.* medium alone; $^{**}p < 0.001$ *vs.* medium alone; §$p < 0.0001$ *vs.* LPS alone **(A)** or respective control **(C-D)**.

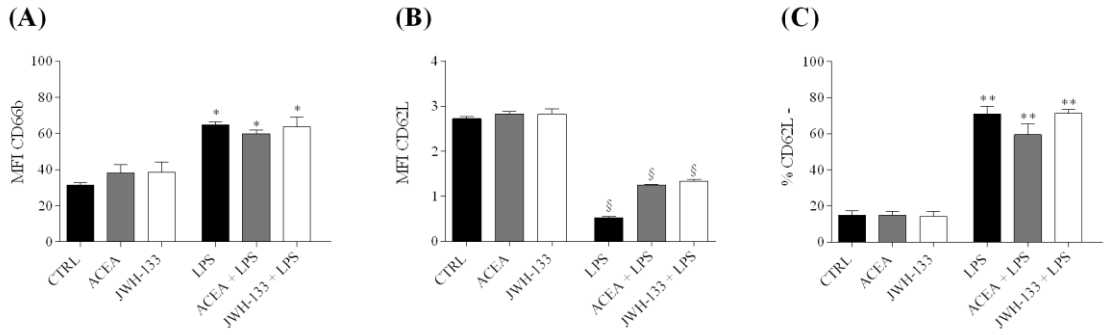


**Figure 7. Effect of ACEA and JWH-133 on CD66b and CD62L expression in LPS-activated PMNs.**

PMNs were preincubated (20 min, 37°C) with or without ACEA (100 nM) or JWH-133 (100 nM) and then stimulated (30 min, 37°C) with LPS (100 ng/ml), stained for PMN activation markers CD66b **(A)**, and CD62L **(B-C)** and subjected to cytofluorimetric analysis. The results were expressed as mean fluorescence intensity (MFI) **(A, B)** or percentages of negative cells **(C)** gated on PMNs. Data are the mean ± SD of 5 experiments.

**p* value ≤ 0.05, ***p* value ≤ 0.01 and §*p* value ≤ 0.001 *vs.* respective control.

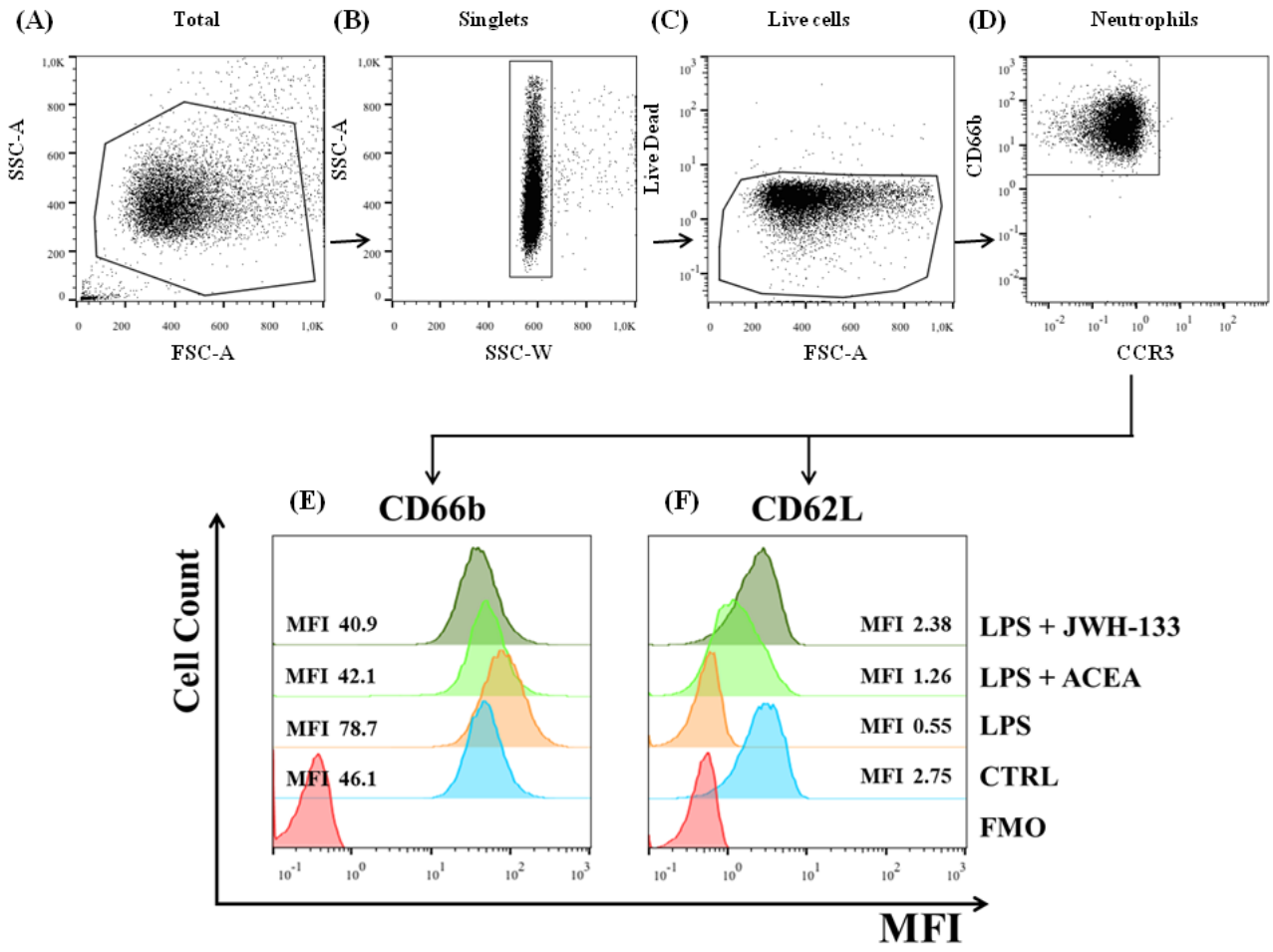


**Figure 8. Effect of ACEA and JWH-133 on CD66b and CD62L expression in LPS-activated PMNs.** Representative flow cytometric panels with respect to the gating strategy of total cells **(A)**, singlets **(B)**, live cells **(C)** and CD66b+ CCR3- neutrophils **(D)**. Representative histograms illustrating mean fluorescence intensity (MFI) for CD66b **(E)** and CD62L **(F)** for one out of 5 independent experiments. FMO = fluorescence minus one.

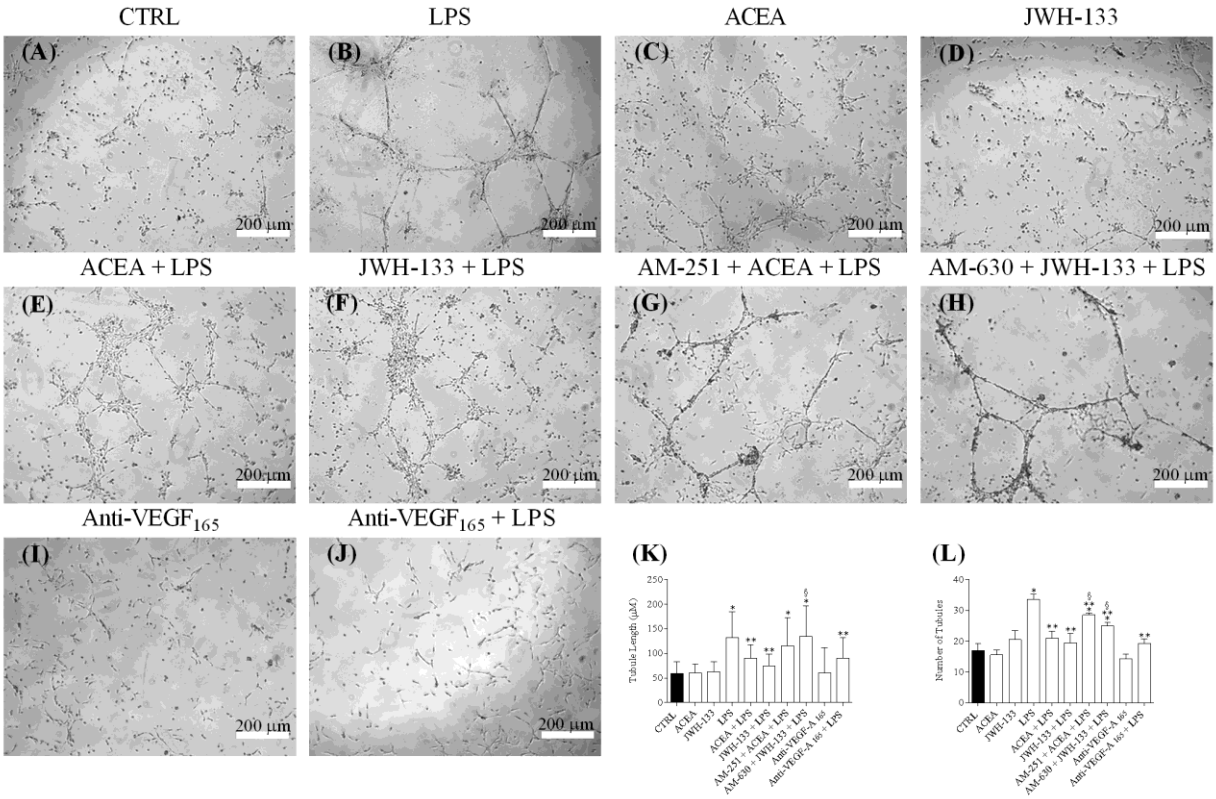


**Figure 9. Effects of $CB_1$ and $CB_2$ agonists on *in vitro* angiogenesis.**

BAECs were plated on Matrigel matrix, and tubular formations were evaluated 6 hrs after treatment with supernatants of PMNs stimulated with **(A)** medium alone, **(B)** LPS (100 ng/ml), **(C)** ACEA (100 nM), **(D)** JWH-133 (100nM), **(E)** ACEA + LPS, **(F)** JWH-133 + LPS, **(G)** AM-251 (500 nM) + ACEA + LPS, **(H)** AM-630 (500 nM) +JWH-133 + LPS, **(I)** anti-VEGF-A (1 μg/ml), **(J)** anti-VEGF-A + LPS. Pictures of tubular formations were obtained using an optical microscope (2.8x).Images are representative of results from 3 independent experiments. The scale bar is 200 $\mu$m. The angiogenic response was quantized as tubule length **(K)** and the number of tubules **(L)** Data in panel K and L are the mean ± SD of 3 independent experiments.

*$p$ < 0.05 *vs.* CTRL; **$p$ < 0.05 *vs.* LPS; §$p$ < 0.05 *vs.* LPS + ACEA or JWH-133 respectively.

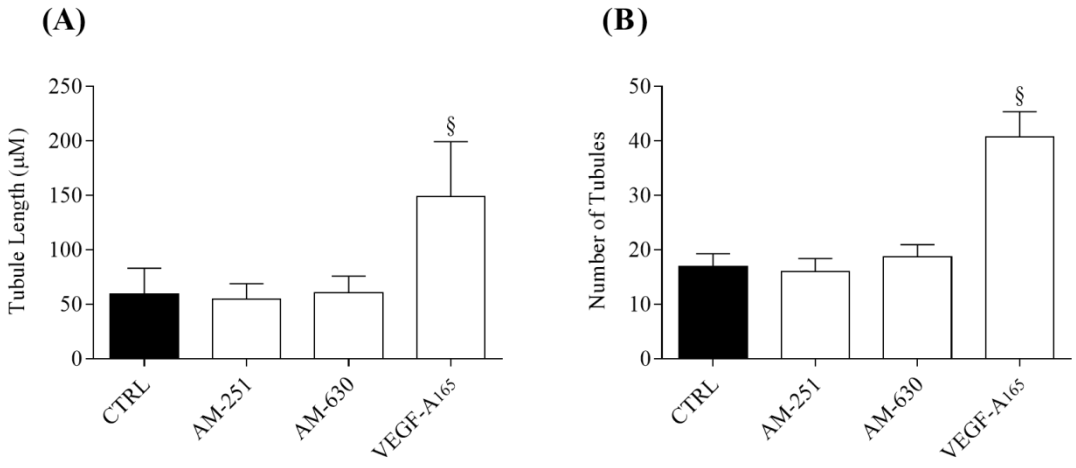


**Figure 10. Effects of $CB_1$ and $CB_2$ antagonists and VEGF-$A_{165}$ on *in vitro* angiogenesis.** BAECs were plated on Matrigel matrix and tubular formations were evaluated 6 hrs after treatment with supernatants of PMNs stimulated with AM-251 (500 nM), AM-630 (500 nM) and VEGF-$A_{165}$ (20 ng/ml). The angiogenic response was evaluated as tubule length **(A)** and the number of tubules **(B)**. Results are expressed as mean ± SD of 3 independent experiments. §$p < 0.005$ *vs.* CTRL.

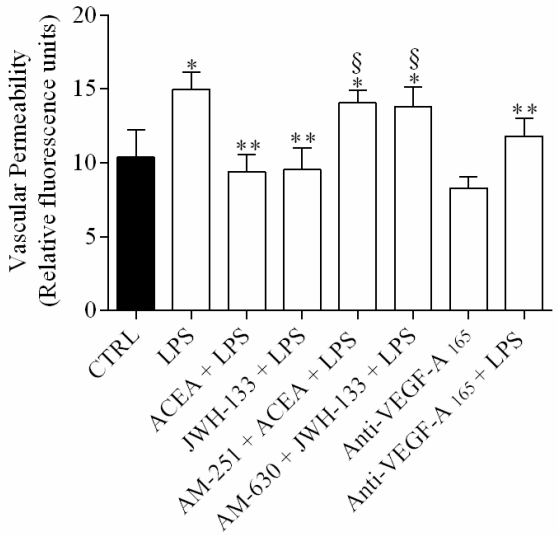


**Figure 11. Effects of $CB_1$ and $CB_2$ agonists on vascular permeability.**

BAEC were incubated (6 hrs, 37°C) with supernatants of PMNs stimulated with medium alone (CTRL), LPS (100 ng/ml), ACEA (100 nM) + LPS, JWH-133 (100 nM) + LPS, AM-251 (500 nM) + ACEA + LPS, AM-630 (500nM) + JWH-133 + LPS, anti-VEGF-A (1 µg/ml) and anti-VEGF-A + LPS. The *in vitro* vascular permeability was assessed as indicated in Materials and Methods. Data are the mean Relative Fluorescence Units (RFU) ± SD of 5 experiments.

*$p$< 0.05 *vs.* CTRL; **$p$ < 0.05 *vs.* LPS; §$p$ < 0.05 *vs*. LPS + ACEA or JWH-133 respectively.

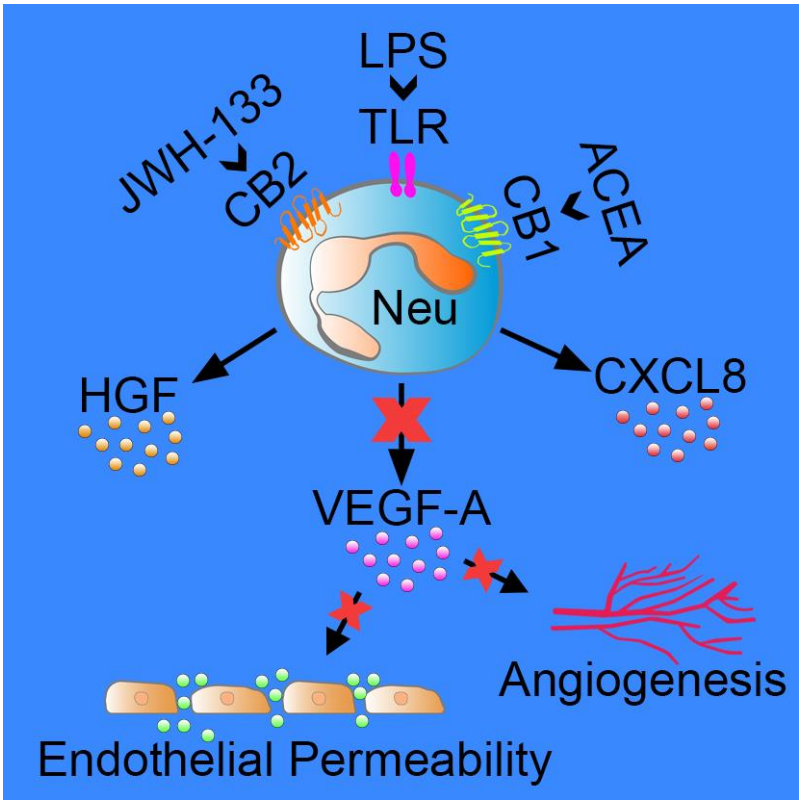


**Figure 12. Schematic representation of the effects of CB agonists on VEGF-A release in LPS-activated neutrophils**